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by Specific Expression of Anti-Apoptotic Molecules in Normal  
Cells

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13. ABSTRACT (Maximum 200 Words)

Adjuvant radiation and chemotherapy confer a survival benefit in breast cancer, but both treatments can damage normal tissues in ways that can adversely affect quality of life (e.g., by skin desquamation, mucositis, pulmonary fibrosis, cardiomyopathy, peripheral neuropathy). These effects on normal tissues are generally due to apoptosis (programmed death) of normal cells. We hypothesize that ectopic overexpression of the anti-apoptotic molecule Bcl-2 will inhibit the radiation-induced apoptosis of normal cells and thus reduce the toxicity of these treatments. We found that overexpressing Bcl-2 in murine fibroblast NIH3T3 cells resulted in resistance to radiation. Heterogeneous plasmid that expresses Bcl-2 cDNA in front of a minimal promoter regulated by multiple wild-type p53 DNA-binding sites protects specifically cells with wild-type p53—but not p53-mutated or p53-deleted cancer cells from genotoxic damage (e.g., radiation) by upregulated expression of p53 and Bcl-2. Progress is described the results of specific aim 1 and 2 that allows to develop preclinical animal model in specific aim 3.

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## Introduction

The toxic effects of radiation and chemotherapy on normal tissues is a significant problem for patients with breast cancer because those effects worsen quality of life (QOL) and hinder the ability to tolerate these conventional therapies at effective levels. Our long-range goals are to reduce the toxicity associated with radiation therapy or chemotherapy and to improve the QOL of patients with breast cancer. One mechanism by which radiation and chemotherapy produce toxic effects is through inducing apoptosis of normal cells in normal tissues. We hypothesize that ectopic overexpression of anti-apoptotic molecules will inhibit the radiation- or chemotherapy-induced apoptosis of normal cells and thereby reduce the toxicity of these treatment modalities. We have shown that overexpression of the anti-apoptotic gene Bcl-2 can protect normal breast epithelial cells from apoptosis *in vitro*, but whether this effect will reduce the toxicity associated with radiation or chemotherapy in patients is unknown. Cells that contain wild-type (wt) p53 typically react to the genotoxic stress of radiation or chemotherapy by upregulating the expression of p53, which binds to specific DNA sequences and activates specific genes, some of which activate apoptosis. To prevent expression of anti-apoptotic genes by cancer cells, we exploited the fact that many types of cancer cells lack or have mutated forms of p53 and are developing a construct in which expression of anti-apoptotic genes is driven a minimal promoter under the control of the wt p53 binding sequence. This strategy is expected to limit the expression of anti-apoptotic genes to normal cells, thus reducing the risk that breast cancer cells become chemo- or radioresistant because of inappropriate overexpression of anti-apoptotic molecules. We expect that cells under genotoxic stress (by being exposed to chemotherapy or radiation) will express higher levels of anti-apoptotic molecules owing to upregulation of p53. Finally, we will use LPD cationic liposomes to create a novel, nonviral gene delivery system for systemic delivery of these anti-apoptotic molecules to normal organs such as lung, liver, kidney, and spleen. This proposal is innovative in that it seeks to prevent or reduce the toxic side effects of conventional therapies (i.e., radiation and chemotherapy) by inhibiting the fundamental biological process of "apoptosis" that they induce in a wt p53-specific manner. The results obtained may lead to the discovery of effective ways to protect normal tissues from radiation or chemotherapy without reducing the efficacy of those treatments.

The specific aims proposed to meet these goals are as follows.

Specific Aim 1. To induce p53-dependent inhibition of radiation- or chemotherapy-induced apoptosis by anti-apoptotic molecules.

Specific Aim 2. To develop a promoter specific for wild-type p53-expressing cells, using wild-type p53-DNA binding sites upstream from a minimal promoter.

Specific Aim 3. To determine the extent to which anti-apoptotic molecules under the control of a novel p53-specific promoter (PGn) complexed with LPD can be used, under optimal conditions, to reduce the toxicity of radiation therapy or chemotherapy.

**The goal for year 2 of the original application was to complete Specific Aim 1 and 2. Progress toward that goal is described in the remainder of this report.**

## Body

### Key Research Accomplishments

**Specific Aim 1.** *To induce p53-dependent inhibition of radiation- or chemotherapy-induced apoptosis by anti-apoptotic molecules.* Our original hypothesis was that upregulation of wt p53 by radiation or chemotherapy will result in overexpression of anti-apoptotic molecules under the control of a minimal promoter downstream of p53 binding sites, which will in turn inhibit stress-induced apoptosis. We addressed this hypothesis in the following 2 subaims.

*Determine whether overexpression of Bcl-2 in normal cells can inhibit apoptosis in vitro and improve survival of cells*

#### Generation of Bcl-2-expressing MDA-MB-231 and NIH-3T3 derivatives

To determine if enforced expression of the Bcl-2 can inhibit radiation induced cytotoxicity, we made Bcl-2 constitutively expressing cells. Monolayers of MBA-MD-231 and NIH-3T3 cells were transfected with the neomycin-selectable expression plasmid containing the human *bcl-2* cDNA (CMV/Bcl-2), and colonies formed under G418 selection were cloned and expanded into cell lines. Protein expression in selected individual cloned *bcl-2* transfectants was examined by Western blotting. All MBA-MD-231 (231 #4 and 231 #5) and NIH-3T3 *bcl-2* cloned transfectants (3T3 #46 and 3T3 #47) expressed high levels of the Bcl-2 protein compared with the parental and neo control transfectants (Fig. 1).

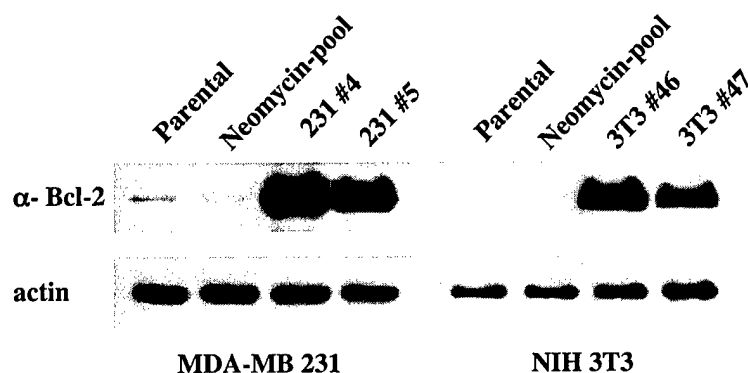


Fig. 1. Detection of Bcl-2 protein expression in MBA-MD-231 and NIH-3T3 cells. Western blot analysis was performed in cell lysates from selected *bcl-2* transfected clones and control cells. The parental cell lines were low Bcl-2 protein expression, while high Bcl-2 protein expression was detected in 2 cloned transfectants analyzed: 231 #4 and 231 #5. NIH-3T3 *bcl-2* cloned transfectants (3T3 #46 and 3T3 #47) expressed high levels of the Bcl-2 protein. Actin was used as a loading control.

## Overexpression of Bcl-2 reduced radiation induced cytotoxicity

To determine if constitutive overexpression of Bcl-2 can inhibit radiation induced cytotoxicity in those cells, we subsequently examined the effect of Bcl-2 over-expression on the cell survival rate after treatment with ionizing irradiation by MTT assay. Significant increase of cell survival was observed after exposure of the parental and neo control cells to a single radiation dose (Fig. 2a, b). These findings indicated that overexpression of Bcl-2 reduced radiation induced cytotoxicity.

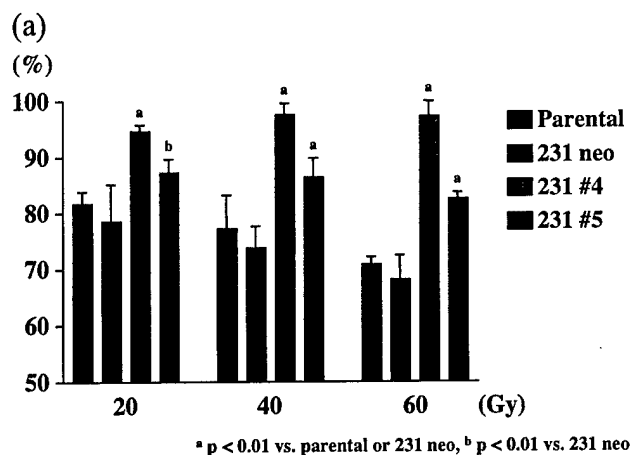
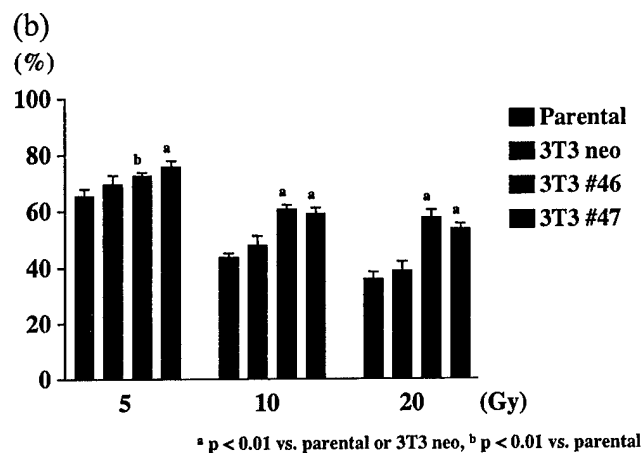


Fig. 2. Bcl-2 reduces radiation induced cytotoxicity. Parental, neo-transfected control and bcl-2-cloned transfectant cells were suspended in 96-well tissue culture plates and preincubated for 24 hr. Cells were exposed to a single dose of ionizing radiation doses of 20 to 60 Gy (a) or 5 to 20 Gy (b), and cell survival rate was determined at 72 hr post-irradiation using the MTT assay. (a) 231 #4 cells were especially resistant to radiation therapy. (b) 3T3 #46 and 3T3 #47 cells showed approximately 20 % resistance to irradiation compared to parental cells.



## Constitutive expression of Bcl-2 reduced apoptosis

To investigate whether Bcl-2 promoted cell survival was due to inhibition of apoptosis, FACS analysis was used to detect the apoptosis following radiation treatment of cultured cells. Figure 3 reveals a % of sub-G1 DNA content of those cells after exposure to single doses of ionizing radiation. Bcl-2-over expressing cells exhibited a significantly reduced apoptosis

compared with the parental of neo control transfectants.

**Therefore, we concluded that Bcl-2 reduces the cytotoxicity of irradiation. All experiments were conducted at least three times. The lab has focused on irradiation more than chemotherapy due to identifying a collaborator who is familiar with irradiation toxicity animal model (Dr. Elizabeth Travis at M. D. Anderson Cancer Center).**

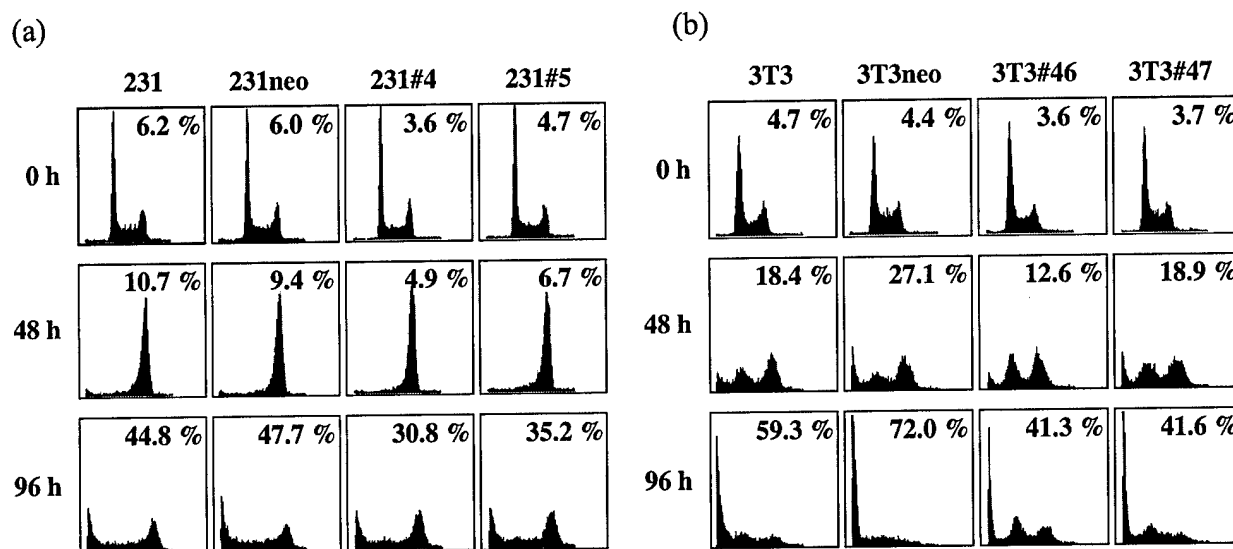


Fig. 3. Constitutive Bcl-2 expression prevents apoptosis induced by irradiation in MDA-MB-231 and NIH 3T3 cells. Parental, neo-transfected control and bcl-2-cloned transfectant cells were suspended in 6-well tissue culture plates and preincubated for 24 hours. Cells were exposed to a single dose of ionizing irradiation doses of 60 Gy (a) or 20 Gy (b). Prior to analysis the cells were washed with PBS, and then resuspended in PBS, containing 40 µg/ml propidium iodide and 0.1 mg/ml RNase. After 30 minutes at 37 °C, the cells were analyzed with a FACScan cytofluorometer, and the population of sub-G1 DNA content was calculated at 48 and 72 hours post-irradiation. (a) 231 #4 and 231 #5 cells were reduced to radiation induced apoptosis. (b) 3T3 #46 and 3T3 #47 cells were reduced approximately 20 – 30 % to radiation induced apoptosis.

***Specific Aim 2. To develop a promoter specific for wild-type p53-expressing cells, using wild-type p53-DNA binding sites upstream from a minimal promoter.***

Our goal is to develop the most efficient promoter that will allow to express Bcl-2 expression in wild-type expressing cells.

**Construction of Bcl-2 expression plasmids with minimal promoter regulated by multiple wild type p53 DNA binding sites**

We constructed p53/Bcl-2 vectors, which contained the polyomavirus early promoter

and bcl-2 gene located downstream of a DNA sequence (PG) that binds wild type (wt) p53 in vitro (Fig. 4a). To test whether wt p53 can induce Bcl-2 protein expression through this vector, we used H1299 lung cancer cells, which have homozygous deletion of the p53 gene. H1299 cells were co-transfected with p53/Bcl-2 and wt p53 vectors. 48 hours after transfection, wt p53 protein strongly upregulated Bcl-2 expression only in p53/Bcl-2 and wt p53 co-transfected cells.

(a)



(b)

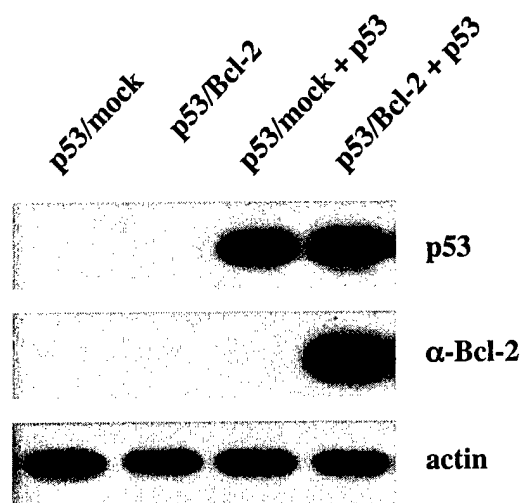


Fig. 4. p53/Bcl-2 vectors were activated by the wild type p53 protein in H1299 cells. (a) Construction of p53/Bcl-2 and p53/mock vectors. PG: the p53 binding sequence; Py: the early gene promoter from polyomavirus (b) H1299 cells were transfected with either p53/mock or p53/Bcl-2 vectors and co-transfected with wild type p53 vectors. Cells were harvested for Western blot analysis 48 hours after transfection and blots were probed for p53, Bcl-2 and actin. The expression of Bcl-2 protein was observed only in p53/Bcl-2 and p53 co-transfected cells. Actin was used as a loading control.

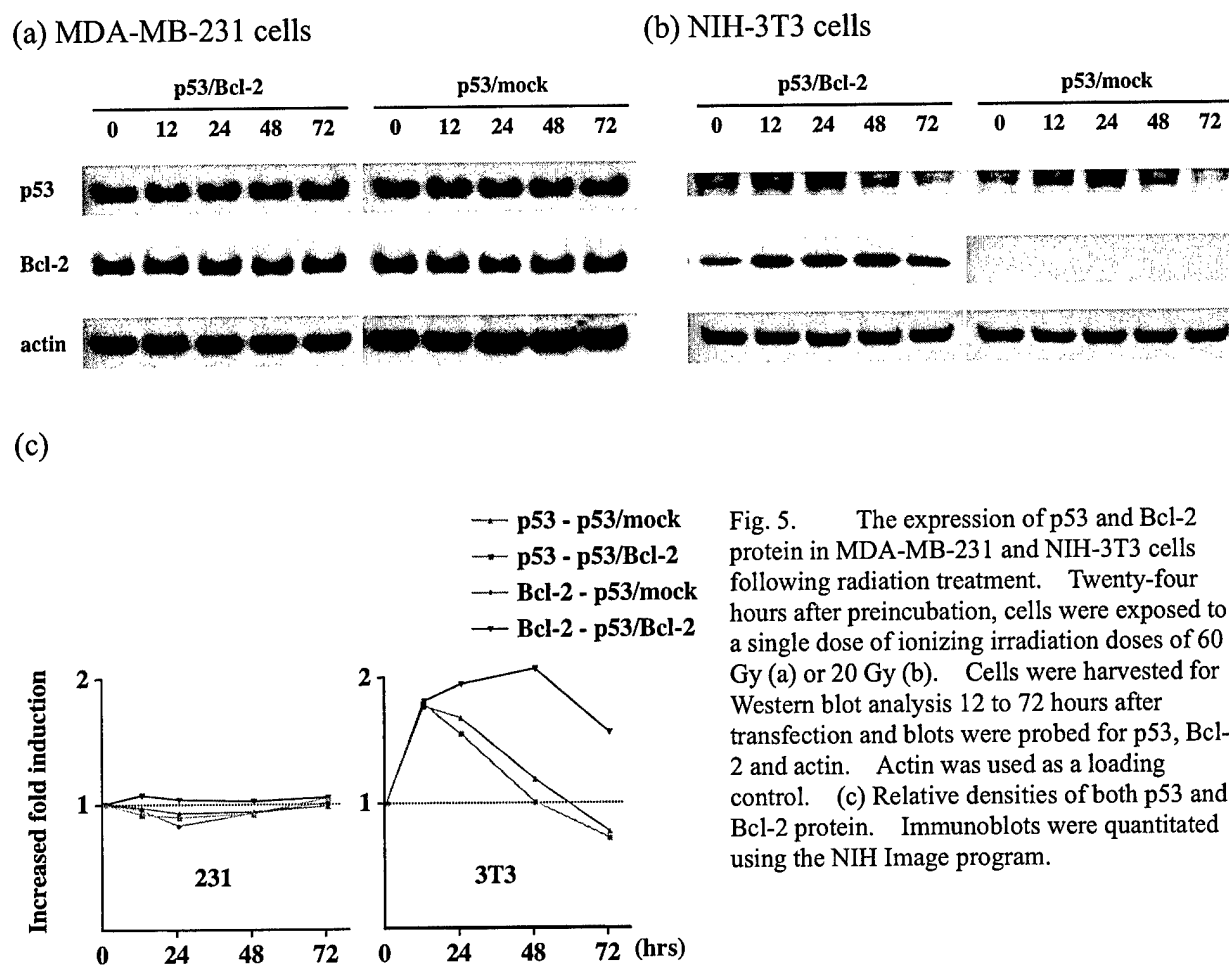
### Ionizing radiation induced wild type p53 can activate p53/Bcl-2 vectors

To test if ionizing radiation induced wild type p53 can activate p53/Bcl-2 vectors, we performed time course study of the expression of p53 and Bcl-2 protein. MDA-MB-231 cells, which has the mutated p53 gene, and NIH-3T3 cells, which has wt p53 gene, were transfected with p53/mock or p53/Bcl-2 vectors. In MDA-MB-231 cells both p53 and Bcl-2 expression were not changed after treated with radiation (Fig. 5a). In contrast, the p53 expression was upregulated 12 hours after treated with radiation in NIH-3T3 cells. And the Bcl-2 expression increased 12 hours after treatment only in p53/Bcl-2 transfected cells (Fig. 5b).



We quantitated the immunoblots using the NIH Image program (Fig. 5c). In MDA-MB-231 cells, both p53 and Bcl-2 expression were not changed after treated with radiation. However, in NIH-3T3 cells, the p53 expression increased 12 to 24 hours after irradiation. And Bcl-2 expression increased in p53/Bcl-2 transfected cells.

These data suggest that NIH-3T3 cells under stress from radiation rapidly upregulated the expression of wt p53, the resulting wt p53 bind to the p53 binding sequence and the transcriptional activity of anti-apoptotic Bcl-2 was increased.



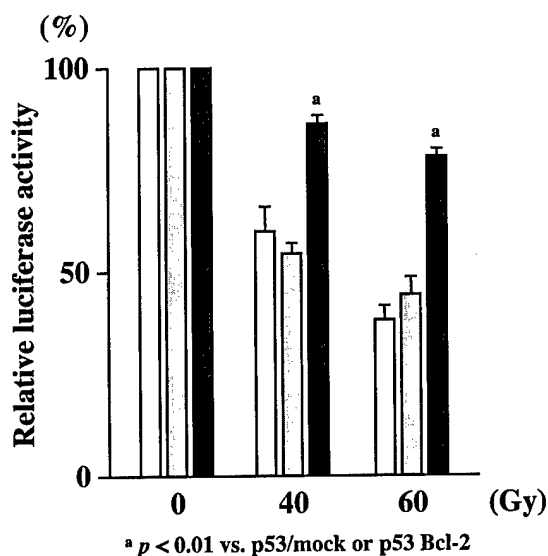
#### p53/Bcl-2 transfected normal cells were reduced radiation induced cytotoxicity

To assess the cell survival rate, MDA-MB-231 cells and NIH-3T3 cells were transfected with p53/mock, p53/Bcl-2 or CMV/Bcl-2 vectors and luciferase vector was co-

transfected in each experiment. At 24 hours post-transfection, cells were treated with radiation. In MDA-MB231 cells, an increased cell survival rate was observed only in CMV/Bcl-2 transfected cells compared to p53/mock or p53/Bcl-2 transfected cells. In NIH-3T3 cells, an increased cell survival rate after irradiation was observed in p53/Bcl-2 or CMV/Bcl-2 transfected cells.

These findings indicated that p53/Bcl-2 transfected cells, which has wt p53, can express the Bcl-2 proteins after treated with radiation and were reduced radiation induced cytotoxicity.

(a) MDA-MB9-231 cells



(b) NIH-3T3 cells

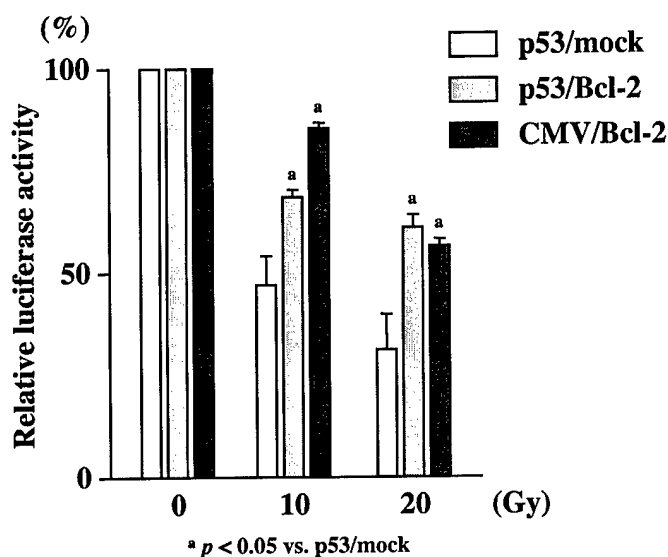


Fig. 6. Activation of p53 induced Bcl-2 and reduced cytotoxicity of ionizing radiation. p53/Bcl-2 and luciferase vectors were co-transfected into MDA-MB-231 cells or NIH-3T3 cells and preincubated for 24 hours. Cells were then exposed to a single dose of ionizing radiation doses of 40 to 60 Gy (a) or 10 to 20 Gy (b). After incubation for 72 hours, the activity of luciferase was measured. The luciferase activity of no irradiated cells were defined as 100 %.

**p53/Bcl-2 transfected normal cells were reduced radiation induced apoptosis**

To detect the apoptosis, MDA-MB-231 cells and NIH-3T3 cells were transfected with p53/mock, p53/Bcl-2 or CMV/Bcl-2 vectors and green fluorescent protein (GFP) vector was co-transfected in each experiment. Ninety-six hours after radiation treatment, we performed dual-parameter analysis of GFP and annexin V-PE. In MDA-MB-231 cells, apoptosis was reduced only in CMV/Bcl-2 transfected cells 96 hours after treatment (Fig. 7a). In NIH-3T3 cells, apoptosis was reduced in p53/Bcl-2 or CMV/Bcl-2 transfected cells (Fig. 7b).

**We concluded that Bcl-2 reduce the cytotoxicity (apoptosis) of irradiation specifically in wild type p53 cells. And irradiation induced wt p53 in normal cells which allow to induce Bcl-2 expression under the control of p53 binding sites.**

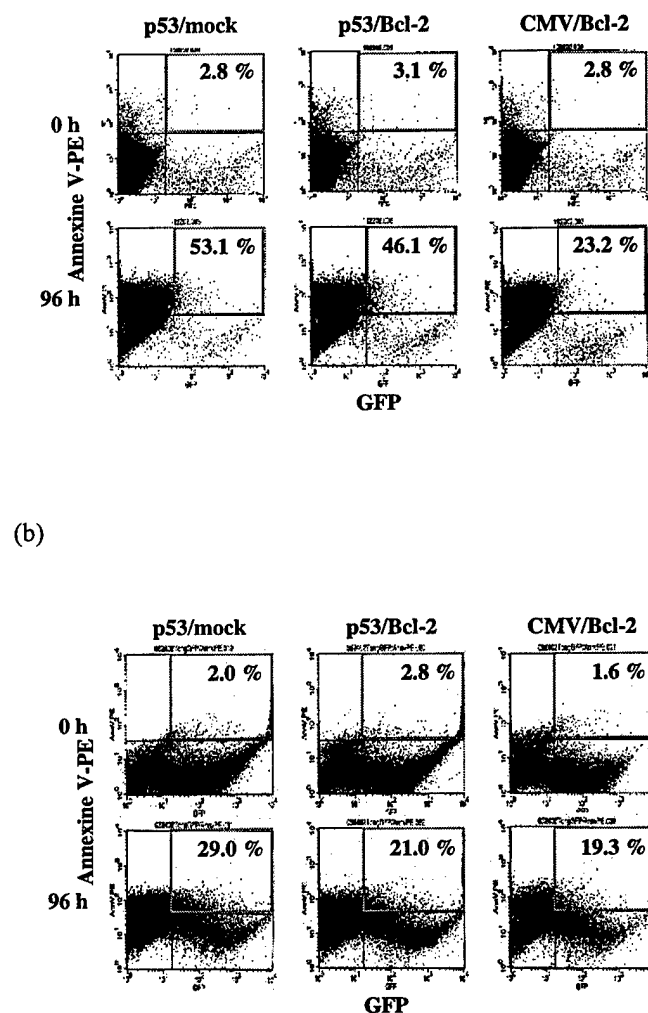


Fig. 7. Detection of apoptosis in MDA-MB-231 and NIH 3T3 cells following radiation treatment. p53/Bcl-2 and GFP vectors were co-transfected into MDA-MB-231 or NIH 3T3 cells and preincubated for 24 hours. Cells were exposed to a single dose of ionizing irradiation doses of 60 Gy (a) or 20 Gy (b). For the detection of phosphatidylserine residues, ANNEXIN V-PE APOPTOSIS DETECTION KIT I was used. Analysis of GFP fluorescence and detection of apoptosis markers were performed on a FACScan cytofluorometer at 96 hours post-irradiation. (a) In MDA-MB-231 cells apoptosis was reduced only in CMV/Bcl-2 transfected cells. (b) In NIH-3T3 cells, apoptosis was reduced in p53/Bcl-2 or CMV/Bcl-2 transfected cells.

**Specific Aim 3 (Reduction of radiation- or chemotherapy-induced toxic effects in normal cells in mice bearing mutated p53 breast cancer xenografts) will be addressed in the third years of funding.**

### **Reportable Outcomes**

The findings described here will be submitted to AACR. We are currently preparing a manuscript that will be submitted to Cancer Research for protection from radiation-induced apoptosis specifically in wild type p-53 cells. This concept allows to justify proceeding with our last specific aim 3.

### **Conclusions**

We have shown that ectopic overexpression of Bcl-2 counters the cytotoxicity of doxorubicin (Adriamycin), paclitaxel (Taxol), and radiation in the first year. In the second year, we showed that Bcl-2 expression can be induced specifically in cells with wild-type p53, in particular under genotoxic stress (irradiation) when cells were transfected with a heterogeneous promoter of a wild-type p53-specific promoter combined with Bcl-2 cDNA. These findings indicate that normal cells expressing wild-type p53, but not p53-mutated or p53-deleted breast cancer cells, can be protected from at least some of the effects of radiation by inducing the wt p53-specific expression of Bcl-2. The concept is now solid to test this heterogenous plasmid in animal mode which will be irradiated to induce mucositis or pulmonary toxicity.

## **References**

**N/A**

## **Appendices**

**N/A**